

## Basic and Translational Research to Understand Factor VIII Immunogenicity

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## **Abstract**

Inhibitor formation against coagulation factor VIII (FVIII) is an unresolved serious problem in replacement therapy for the X-linked bleeding disorder hemophilia A. Although FVIII inhibitors have been extensively studied, much of the basic mechanism of this immune response remains to be uncovered. Within the NHLBI State of the Science Workshop on Factor VIII Inhibitors, Working Group 3 identified three scientific priorities for basic and translational research on FVIII inhibitor formation. These include activation signals and immune regulation that shape the response to FVIII (including innate immunity, microbiome, adaptive immunity and regulatory T cell studies in humans); utility of animal models and non-animal approaches (in silico, genetic, single cell/sorted population 'omics, in vitro) to help predict inhibitor formation and identify novel therapeutics; and impact of the source of FVIII, its structure, and von Willebrand factor on immunogenicity and tolerance. Early interactions between FVIII and the immune system, biomarker development, and studies in different patient groups (previously treated or untreated, with or without inhibitor formation, patients undergoing immune tolerance induction or gene therapy) deserve particular emphasis. Finally, linking basic to clinical studies, development of a repository for biospecimens, and opportunities for interdisciplinary research training are important components to solving the urgent problem of inhibitor formation.

## INTRODUCTION

Inhibitor formation against coagulation factor VIII (FVIII) is an unresolved serious problem in replacement therapy for the X-linked bleeding disorder hemophilia A <sup>1</sup>. Although FVIII inhibitors represent the perhaps best studied example of anti-drug antibody formation, much of the basic mechanism of this immune response remains to be uncovered. Importantly, such basic questions provide guidance for translational and clinical studies. For instance, one may be able to devise strategies that reduce the incidence of inhibitor formation, better predict which patients are likely to form inhibitors or to tolerize after initial inhibitor development, or develop biomarkers for early stages of a developing immune response or, conversely, tolerance.

It is known that the activation of B cells that leads to the production of inhibitors requires help from CD4<sup>+</sup> T cells (**Fig. 1**). Critical co-stimulatory pathways for dendritic cell (DC) – T cell and B cell – T cell interactions include CD86-CD28, ICOS-ICOSL and CD40-CD40L <sup>2</sup>. Regulatory T cells (Tregs) are able to suppress antibody formation. The role of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs in tolerance to FVIII has been described in numerous studies, while latency associated peptide (LAP) expressing Tregs are more typical in oral tolerance to FVIII, representing various approaches to achieve tolerance <sup>3-6</sup>. Presumably, innate immune signals that may derive from tissue damage or molecular patterns associated with pathogens trigger initiation of these adaptive immune responses. For example, bacterial lipopolysaccharide (LPS) functions as an adjuvant and enhances FVIII inhibitor formation in hemophilic mice. Certain types of macrophages and marginal zone B cells have also been implicated in initial FVIII uptake in lymphoid organs <sup>7,8</sup>. How these innate factors and cell types [including innate immune and antigen presenting cells (APCs)] interact during the transition from initial innate immune signaling to the adaptive immune response remains to be defined.

Liver sinusoidal endothelial cells (LSEC) are the predominant natural site of FVIII biosynthesis, although other endothelial cells (such as lymphatic and other fenestrated endothelium) also contribute <sup>9-11</sup>. Recombinant FVIII is made in various mammalian cell lines

(none of which are endothelial cells), and current gene therapies target hepatocytes for FVIII expression. The latter can induce immune tolerance, although recent literature suggests that targeting LSEC, as compared to hepatocytes, may better induce tolerance, perhaps owing to their ability to promote induction of Treg<sup>5,6,12</sup>.

Potential reasons for differences in immunogenicity of FVIII produced by varying cell types need to be better addressed. Interestingly, FVIII produced in cultured cells with different N-glycosylation showed differences in inhibitor formation in hemophilic mice<sup>13</sup>. Von Willebrand Factor (vWF), which binds to FVIII and is critical for its half-life in the circulation, has been shown in several studies to affect FVIII immunogenicity, possibly by changing APC and/or endothelial cell FVIII uptake<sup>14</sup>. Receptors on macrophages and sinusoidal endothelial cells, such as the recently identified stabilin-2, regulate this process<sup>15</sup>. Thus, the structure of FVIII (including posttranslational modifications), its interactions with other plasma proteins and cell surface receptors, and its site of synthesis all impact its immunogenicity; but, there is more to be learned about these different mechanisms and how they interact. Recent autoimmune disease studies documented that the gut microbiome can also play a role in regulating antibody formation<sup>16</sup>. Antigens in the systemic circulation are also taken up by APCs in the gut associated immune system. The role of the microbiome in immunity to FVIII has not yet been determined.

There has been progress in the identification of risk factors for inhibitor formation, including polymorphisms in the promoter regions of genes related to immune functions<sup>17</sup>. The underlying F8 mutation is an expected factor since greater loss of coding information reduces the likelihood of central tolerance. How much the major histocompatibility complex (MHC) contributes is less clear, with some studies however providing evidence for a correlation between MHC and inhibitor formation. Several CD4<sup>+</sup> T cell epitopes (and the HLA-class II molecules that present them) have been identified. Some studies suggest a large epitope repertoire, while others point to a more restricted set of epitopes that actually drive the T cell response during inhibitor formation<sup>18,19</sup>. Substantially more information about genetic factors is expected to emerge from the nationwide

My Life Our Future initiative ([www.mylifeourfuture.org](http://www.mylifeourfuture.org)), which already resulted in the genotyping of several thousand patients with hemophilia in the US <sup>20</sup>.

It is noteworthy that multiple animal models are available for pre-clinical studies in hemophilia <sup>21</sup>. These include hemophilia A mice with targeted deletions of F8 exons 16 or 17, bred on different genetic strain backgrounds, in addition to a total gene deletion hemophilia A mouse on a C57BL/6 background. Transgenic hemophilia A mice expressing certain human HLA molecules are also available. Hemophilia A dogs, e.g. with a F8 intron 22 inversion analogous to the most common mutation in humans, are a well characterized large animal model. Hemophilic animals of other species have also been identified but not as widely studied.

## **MATERIALS AND METHODS**

### *Working group membership:*

A group of 17 individuals with scientific expertise in diverse areas were assembled (see **Table 1**). Their areas of research and expertise included FVIII biochemistry, adaptive and innate immunity, immune regulation and tolerance, product development, in silico modeling, systems biology, animal models, genetics, genomics, clinical research, gene therapy, and microbiome studies, as well as patient treatment and advocacy.

### *Plan of execution:*

The Working Group initially identified a large number of potential goals for basic and translational research on FVIII inhibitor formation and then developed a strategy for scientific prioritization. Ultimately, 3 scientific priorities were identified. Subgroups were formed to develop the details of each priority. Subsequently, the entire Working group weighted each scientific goal based on required effort and potential impact and identified approaches, methods/technologies, and models to complete the studies. Strategies to connect and embed mechanistic research into clinical studies and clinical trials were discussed. Requirements and strategies for biospecimen

collection and distribution were developed. Finally, opportunities for interdisciplinary research training were identified.

## RESULTS AND DISCUSSION

### Scientific priorities and implementation strategies for basic research:

Significant gaps exist in the knowledge of the mechanism of the immune response to FVIII, and multiple factors affect FVIII immunogenicity. Therefore, tackling this complex scientific problem requires scientific priorities, and implementation of strategies for acquiring an actionable understanding of FVIII immunogenicity and the biology of both host immunity and tolerance. To aid in the determination of scientific prioritization, different aspects of potential research goals were evaluated by Working Group 3 for their potential impact to the field relative to the degree of effort and cost necessary. Similarly, the advantages and limitations of human and model studies (animal, cell, *in silico*) were taken into consideration. Finally, the sequence of research pathways was addressed. For example, for human studies, genetic and immune characterization studies are low effort but potentially high impact when analyzing samples from previously treated patients (PTPs), for whom blood draw volumes are less limiting. These studies need to be performed prior to studies in previously untreated patients (PUPs) to help determine best use of limited sample volumes (**Fig. 2**). It should be recognized that immunological studies in the 70-80% of PTP who have not formed inhibitors are similarly important to immune response mechanisms in inhibitor patients, because these will help delineate why the response is directed to tolerance or antibody formation, aid in the identification of biomarkers, and potentially lead to interventions that favor immune tolerance induction and thus prevent inhibitor formation. Such studies will also improve prediction of risk of inhibitor formation for individual patients.

### Scientific priorities for basic and translational research on FVIII immunogenicity:

Ultimately, the scientific priorities for basic and translational research were divided into 3 priorities that approached immunity to FVIII from two aspects: (1) mechanisms of the initial

immune response upon FVIII exposure that results in peripheral tolerance or inhibitor development and (2) mechanisms by which the immune system responds to FVIII exposure with immune tolerance induction (ITI) following initial inhibitor development. The latter may be based on traditional ITI protocols or emerging gene therapies. In addition, potential modifiers of the immune response, such as the microbiome, glycosylation patterns, and route of FVIII exposure (intravenous, subcutaneous, cellular production following gene therapy) were considered. Specifically, the following three scientific priorities were identified: (1) Activation signals and immune regulation that shape the response to FVIII (including innate immunity, microbiome, adaptive immunity and Treg studies in humans; **Table 2**); (2) Utility of animal models and non-animal approaches (*in silico*, genetic, omics, *in vitro*) to help predict inhibitor formation and identify novel therapeutics (including single cell approaches; **Table 3**); and (3) Impact of the source of FVIII, its structure, and vWF on immunogenicity and tolerance (including the impact of gene therapy on FVIII tolerance versus immunogenicity, considering the choice of vector, transgene and cellular target; **Table 4**).

Within priority #1, particular emphasis should be placed on early interactions between FVIII and the immune system, which are shaped by innate immune cells, signals from tissue and potentially microbiome, and FVIII structure/biochemical aspects. Furthermore, biomarkers that can be used to detect the immune response early and that correlate with a subsequent adaptive response, resulting in inhibitor formation, are currently lacking.

Study designs to address these priorities would need to consider several key questions: What are the critical genes and pathways that shape the immune response to FVIII (including innate immunity and microbiome effects), and how can they be targeted to reduce immunogenicity to FVIII? How can non-animal approaches be used to predict and model inhibitor formation and tolerance induction? What are the advantages and disadvantages of different animal models? For example, one may be able to develop or incorporate additional mouse strains into model studies to assess genetic modifiers of the immune response. How does the site of FVIII expression, its

structure, and vWF determine immunogenicity and tolerance? How can this knowledge be applied to gene therapy and to the selection of strategies to induce immune tolerance? What experimental models and novel technologies are available and being used by groups such as the Immune Tolerance Network (ITN)? How can they be utilized to investigate FVIII immunogenicity and mechanisms of tolerance? These latter questions lead to another critical aspect in study design, the incorporation of basic research into clinical and translational studies in order to maximally benefit patients.

*Models for the integration of basic science into clinical studies/translational research:*

Studies on immune mechanisms can be integrated into clinical antenatal/neonatal cohort studies, into lifespan studies on inhibitor development, and into clinical trials, including accompanying biospecimen procurement that ideally would be handled by a central biorepository and processing center<sup>22-24</sup>. This center would do as much of the processing on site as possible using standardized protocols and disperse samples to different investigators to minimize processing inconsistency. Ability to link the respective specimens to key clinical events will greatly enhance their utility. The framework and timing of biospecimen procurement should be informed by the scientific priorities and then integrated into the different trials and observational studies to maximize the scientific data that can be obtained from a limited number of subjects. Because of the relatively small number of PUPs, and the challenges of numbers needed for adequate statistical power, it may be advantageous to implement a collaborative network with independent oversight from an expert steering committee. Such a structure could then triage and prioritize basic research questions and facilitate the collaborative distribution of samples from the limited pool of patients. A possible model for such a design is the APOLLO (APOL1 Long-term Kidney Transplantation Outcomes Network) network and studies funded by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). All participating sites would be required to pursue independent research while contributing DNA or other biological samples to the



consortium from their site or a partner clinical site. Individual clinical centers that receive funding from other community studies would be expected to contribute as many types of samples as possible given their available resources. Given recent advances in gene therapy as a potential cure for hemophilia, it would be most advantageous to also incorporate analysis of immune responses to FVIII in gene therapy trials and work, potentially with industry partnerships, toward a clinical trial on reversal of inhibitors by gene therapy (**Fig. 2**)<sup>22</sup>. This will also help prepare gene therapy trials that aim to induce tolerance in patients with inhibitors. Again, an important aspect of clinical development of any novel approach to eradication of inhibitors will be identification of biomarkers for tolerance induction to FVIII.

For any combined basic science/clinical study, the requirements and timelines for collection of biospecimens are important. For instance, a feasible schedule for blood sample collection in PUPs could be prior to and one day following initial FVIII exposure, followed by periodic sampling during the first 50 exposure days and then further albeit less frequent sampling throughout the lifespan. Samples from PTPs should include those who never developed an inhibitor, have a persistent inhibitor, or were successfully tolerized after inhibitor formation. Samples from inhibitor patients should be collected at the time of inhibitor formation, prior to initiation of ITI, 2-4 weeks after starting ITI, and then at regular intervals, such as every 3 months until tolerized. For microbiome studies, fecal samples from mothers should be collected at the end of the first, second and third trimester for 16S or metagenomics analysis. Ideally, the placenta should also be analyzed after birth (**Fig. 3**). As soon as possible after birth, meconium should be collected. Feces from the infant should then ideally be sampled every month in the first year after birth. Any factor that might affect the microbiome should be noted. Antibiotic use in the mother or infant, breast milk versus formula diet, and post-natal exposure to pets or farm animals are among the most important. Though vaginal versus C-section delivery is important, it is a controversial topic.

### Challenges and models for cross-disciplinary research:

Advancing basic science and clinical immunology in the field of FVIII inhibitor formation would greatly benefit from recruitment of scientists from various disciplines, including but not limited to biochemistry, genetics, genomics, bioinformatics, and immunology (**Fig. 4**). The implementation of such interdisciplinary research is always a challenge. A recent example of a model for this type of collaborative research is the NHLBI U54 FVIII Centers initiative ([grants.nih.gov/grants/guide/rfa-files/RFA-HL-18-014.html](https://grants.nih.gov/grants/guide/rfa-files/RFA-HL-18-014.html)) that allow training opportunities in newly established cross-disciplinary settings. These three centers will characterize the functional repertoire and ontogeny of FVIII humoral immunity across species, study the *in vivo* mechanisms of FVIII immunity and the influence of the host microbiome; define the structural basis for FVIII immune recognition and related immunobiology, and study the immunopharmacology of FVIII bioengineering and gene therapy; study the role of glycosylation in inhibitor formation, and characterize genetic effects on FVIII glycosylation patterns and inhibitor formation. Importantly, these centers include skill development cores that aid in the training of interdisciplinary scientists that will be well equipped to develop future studies on immunogenicity of FVIII and tolerance.

For other efforts, such as clinical trials, industry support is critical. However, how to integrate industry goals with core scientific goals remains a challenge. However, an organization such as the NIH Foundation could facilitate industry's support of basic scientific research goals. Regardless of funding opportunities and the structures in place, consistent engagement of scientists whose primary focus is in other areas will require strong partnership between FVIII researchers and these colleagues. Only then one can meet the challenges of keeping up with rapidly changing technology across many diverse fields, ranging from engineering to genetics to immunology.

## ACKNOWLEDGEMENTS

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**Table 1. Members of Working Group 3 organized by their respective expertise.**

<b>Table 1 Working Group 3 Members</b>		
<b>Expertise</b>	<b>Investigator</b>	<b>Affiliation</b>
Molecular Biology & Omics	Cheryl Winkler, PhD	NCI
Factor VIII Biochemistry	Peter Lenting, PhD Peter Lollar, MD	INSERM, Paris Emory University
Immunology	Bernard Khor, MD, PhD Kate Pratt, PhD	Benaroya Research Institute Uniformed Services University
Antigen Generated Peptide Expertise	Betty Diamond, MD Jean Marie Saint-Remy, MD, PhD	Feinstein Institute for Medical Research KU Leuven, Belgium
Gene Therapy Animal and Ex Vivo Models	Valder Arruda, MD, PhD Wadie Bahou, MD David Lillicrap, MD Roland Herzog, PhD	Children's Hospital of Philadelphia Stony Brook University Queen's University, Kingston, ON Indiana University
Microbiome	Josef Neu, MD	University of Florida
<i>In Silico</i> Protein Modeling	Yifan Song, PhD	Cyrus Biotechnology
Drug Development	David Wraith, MD, PhD	University of Birmingham, UK
Industry	Steve Arkin, MD	Pfizer
Patient Community & Advocacy; Treatment	Glenn Pierce, MD, PhD Shannon Meeks, MD	Scientist, NHF, WFH Emory University

**Table 2: Scientific priority #1: Activation signals and immune regulation that shape the response to Factor VIII (FVIII)**

<b>Scientific priority #1: Activation signals and immune regulation that shape the response to FVIII</b>		
<b>Overall study goals</b>	<b>Detailed study goals</b>	<b>Effort/Impact</b>
Innate/early immune recognition of FVIII	Studies in murine models: <ul style="list-style-type: none"> <li>• Side-by-side comparison of mice of different strain backgrounds</li> <li>• Parameters that affect the response, such as glycosylation patterns or microbiome</li> <li>• Biomarker development</li> <li>• Mouse model development (e.g. mice with human immune system)</li> </ul>	High/high
	Human studies: <ul style="list-style-type: none"> <li>• Comparison of innate immunity parameters in patient subpopulations</li> <li>• Biomarker development (cytokines, cellular, expression signatures)</li> <li>• Flow panels to include both activating and regulatory B cell (Breg) and T cell(Treg) phenotypes, such as Th2 or Th17 cells that might be seen in allergic or autoimmune responses</li> <li>• Maternal microbiome studies</li> <li>• Studies early in life through first year after birth</li> <li>• Evaluation of role of antibiotics and breast feeding</li> </ul>	Very high/high
Adaptive immune responses	Murine models: <ul style="list-style-type: none"> <li>• Diverse group of genetic backgrounds with the same F8 mutation in mice to study genetic effects</li> <li>• Improved (humanized) models</li> <li>• Animal model of ITI is desirable</li> </ul>	Modest/high
	Human studies: <ul style="list-style-type: none"> <li>• Immune phenotyping by flow cytometry; biomarker development</li> <li>• Improved T cell epitope mapping</li> <li>• Activation markers in response to FVIII; transcriptome and single cell footprints</li> <li>• Development of technologies to analyze small sample sizes or rare cells such as circulating memory B cells and long-lived plasma cells</li> <li>• Biomarker development (high/high cellular, expression signatures, FVIII-specific immunoglobulins subclasses)</li> </ul>	High/high
Immune regulation/tolerance	Human and animal studies: <ul style="list-style-type: none"> <li>• Biomarkers of effective immunotherapy with FVIII</li> <li>• Gene expression signatures in antigen-specific T cell populations during immunotherapy</li> <li>• Novel methods for tolerance induction</li> <li>• Experimental models for assessing tolerance induction</li> <li>• Treg characterization and potentially other regulatory cells such as Bregs and M2 IL10 producing DCs</li> <li>• Partnership with organizations such as Immune Tolerance Network (ITN)</li> </ul>	Modest to high/high

**Table 3: Scientific priority #2: Utility of non-animal approaches (*in silico*, genetic, omics, *in vitro*) to help predict inhibitor formation**

<b>Scientific priority #2:Utility of non-animal approaches (<i>in silico</i>, genetic, omics, <i>in vitro</i>) to help predict inhibitor formation</b>		
<b>Overall study goals</b>	<b>Detailed study goals</b>	<b>Effort/impact</b>
<i>In vitro</i> assays for antigen presentation and T cell activation	<ul style="list-style-type: none"> <li>• FVIII antigen presentation assays</li> <li>• Novel methods for TCR, BCR repertoire analysis</li> <li>• Mapping of T-cell and B-cell epitopes (experimental and <i>in silico</i> predictions)</li> <li>• Antigen-specific responses of Tregs as well as T-effectors</li> <li>• Analogies to both autoimmune disorders and vaccine studies</li> <li>• Roles of anergy, deletion, Treg in initiation of anti-FVIII immune response and in ITI-refractive patients</li> <li>• Coordination with investigators doing animal model studies</li> <li>• Natural history/phenotyping and potential targets for peripheral tolerance: CD4<sup>+</sup> T cells, long-lived plasma cells, memory B cells</li> </ul>	Modest/modest-high
Genomic, proteomic, transcriptome approaches to phenotype the immune response to FVIII	<ul style="list-style-type: none"> <li>• Genomics studies within MLOF projects</li> <li>• Transcriptomics studies on whole-blood RNA and RNA from defined subsets of cells, such as CD4<sup>+</sup> T cells, including single-cell analyses.</li> <li>• TCR/BCR repertoires of single cells</li> <li>• Proteomics studies to quantify protein expression levels and identify/quantify post-translational modifications; improved proteomic profiling of serum, plasma, whole blood, and cell lysates</li> <li>• Epigenomics studies on sorted cell populations</li> </ul>	High/high
Bioinformatics and <i>in silico</i> modeling of antigen presentation	<ul style="list-style-type: none"> <li>• Establish collaborations between bioinformatic experts and immunologists</li> <li>• Risk factors for failure to respond to ITI may be identifiable by retrospective biostatistical analysis</li> <li>• <i>In silico</i> predictions of epitopes currently have limitations, but can still be useful provided that larger and more quantitative data sets can be obtained</li> <li>• Determining the total number of clinically relevant epitopes would be desirable, although polymorphisms/mutations in HLA result in patient-specific effects</li> <li>• Deimmunization strategies (i.e. removal of immunogenic epitopes) will benefit from expanded epitope mapping and predictions</li> </ul>	Modest effort/unclear impact *there was much debate within WG3 about pros/cons of this approach, with strong opinions that it would be high impact and strong opinions it would be low impact

**Table 4: Scientific priority #3: How does the site of Factor VIII (FVIII) expression, its structure, and von Willebrand factor (vWF) determine immunogenicity and tolerance**

<b>Scientific priority #3:How does the site of FVIII expression, its structure, and vWF determine immunogenicity and tolerance</b>		
<b>Overall study goals</b>	<b>Detailed study goals</b>	<b>Effort/impact</b>
FVIII expressed in gene therapy	<ul style="list-style-type: none"> <li>• More detailed immunological studies in patients participating in current and future gene therapy trials are desirable (e.g. evidence for immune regulation or small, non-clinically evident changes in the immune response to FVIII)</li> <li>• Work toward gene therapy trials in inhibitor patients, in particular those who failed ITI</li> <li>• Define innate immunity to AAV and lentiviral vectors</li> <li>• Study immunogenicity of altered (bioengineered) FVIII molecules that are considered for gene therapy</li> </ul>	High/high
FVIII interactions with vWF	<ul style="list-style-type: none"> <li>• Effect of FVIII molecules with altered interactions with vWF</li> <li>• Modulation of antigen uptake, presentation and clearance through scavenger receptors by vWF</li> <li>• Effect of de-targeting from endogenous vWF in new products currently in development</li> <li>• Development of molecules with more stable binding to vWF</li> </ul>	High/modest to high
FVIII molecules with altered structure	<ul style="list-style-type: none"> <li>• Immunoglobulin can have a tolerogenic effect Therefore, Fc fusion proteins may aid in tolerance induction, e.g. during ITI or <i>in utero</i></li> <li>• Study a potential influence of PEGylation on FVIII immunogenicity</li> <li>• Assess the immunogenicity of engineered FVIII molecules, in particular those with amino acid changes compared to traditional recombinant products</li> </ul>	Modest/high



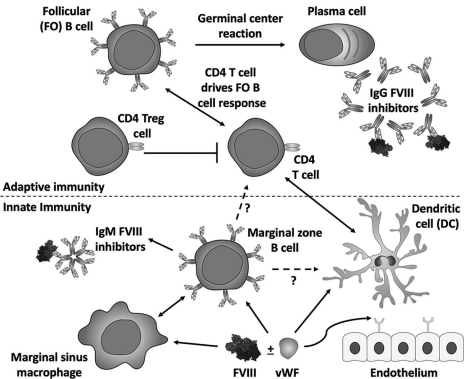
## Figure Legends:

**Figure 1:** Mechanisms of antibody formation against FVIII. Innate immune signals (lower half) may lead to activation of FVIII-specific CD4<sup>+</sup> T helper cells, and ultimately in B cell activation and formation of inhibitors. Long-term inhibitor production may occur in plasma cells, while regulatory T cells (Treg) have the capacity to down-regulate the response and promote tolerance.

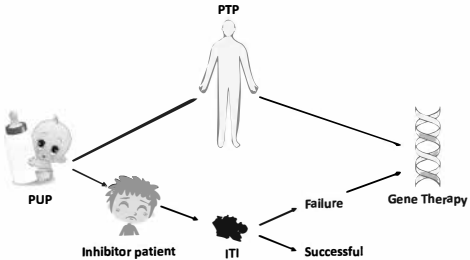
**Figure 2:** Comprehensive collection of blood samples in various hemophilia A patient populations undergoing FVIII replacement therapy; including previous untreated patients (PUPs), previously treated patients (PTPs), patients with and without inhibitors, patients undergoing immune tolerance induction (ITI), and patients being treated with gene therapy.

**Figure 3:** Potential timelines for collection of microbiome samples from pregnant mothers and newborns/infants with hemophilia A. Child development cartoon adopted from *Pediatr Rev.* 1997;18:224–242.

**Figure 4:** Integration of multiple scientific disciplines and approaches to study FVIII immunogenicity.



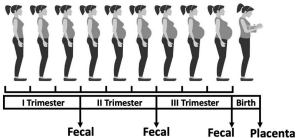
**Fig. 1**



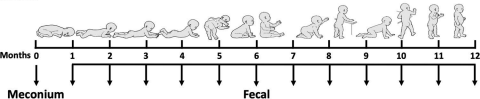
**Fig. 2**

## Microbiome studies - Timelines

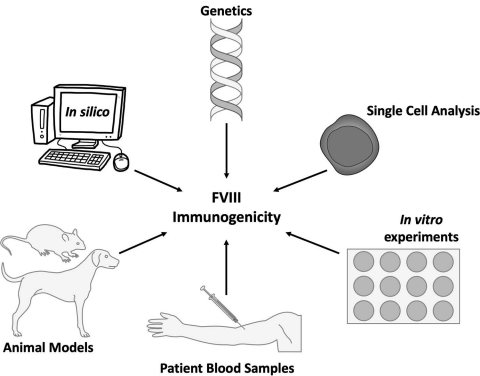
**Maternal:**



**Infant:**



**Fig. 3**



**Fig. 4**